

L-PHENYLALANINE ACTIVATES CLASS A ORPHAN G PROTEIN-COUPLED
RECEPTORS
BY
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Glossary of Abbreviations and Symbols

7TM- Seven trans-membrane

AADC- Aromatic L-Amino Acid Decarboxylase

ADHD- Attention Deficit- Hyperactivity Disorder

ADMET- Absorption, Distribution, Metabolism, Excretion, and Toxicity

AMP- Ampicillin

ANOVA- Analysis of Variance

BRET- Bioluminescence Resonance Energy Transfer

cAMP- Cyclic Adenosine Monophosphate

CaSR- Calcium-Sensing Receptor

CCK- Cholecystokinin

CDS- Coding Sequences

CNS- Central Nervous System

DMEM- Dulbecco's Modification Eagle's Medium 1X Mod.

DMSO- Dimethyl Sulfoxide

DRC- Dose-Response Curve

E- Essential

EDTA- Ethylenediaminetetraacetic Acid

ERK- Extracellular signal-regulated kinases

FBS- Fetal Bovine Serum

GDP- Guanosine diphosphate

GEF- Guanine-Nucleotide Exchange Factor

GFP- Green Fluorescent Protein

GI tract -Gastrointestinal Tract

GPCR- G Protein-Coupled Receptor

GRK- G Protein-Coupled Receptor Kinases

GTP- Guanosine Triphosphate

HCS- High Content Screening

HEK293 cells- Human Embryonic Kidney 293 cells

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HTLA cells- Modified (HEK293) cell line stably expressing tTA-dependent luciferase reporter gene and a β -arrestin2-TEV fusion gene

HTS- High-Throughput Screen

IUPHAR- International Union of Basic and Clinical Pharmacology

L-Arg- L-Arginine

L-DOPA- L-Dihydroxyphenylalanine

L-Phe- L-Phenylalanine

L-Trp- L-Tryptophan

L-Tyr- L-Tyrosine

Min- Minutes

MSN- Medium Spiny Neurons

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NALs- Neutral Allosteric Ligands

NAMs- Negative Allosteric Modulators

NE- Non-Essential

P/S- Penicillin-Streptomycin

PAH- Phenylalanine Hydroxylase

PAMs- Positive Allosteric Modulators

PBS- Phosphate Buffered Saline

PEA- Phenylethylamine

PEI- Polyethylenimine

PEPT1-Peptide Transporter 1

PKU- Phenylketonuria

PRESTO-Tango- Parallel Receptor-ome Expression and Screening *via* Transcriptional Output-
Tango

RGS- Regulators of G Protein Signaling

RLU- Relative Luminescence

RTK- Receptor Tyrosine Kinases

SEM- Standard Error of the Mean

T1R- Taste Receptor Type 1

TEV- Tobacco Etch Virus

tTA- Tetracycline Transactivator

V₂R- Vasopressin type-2 receptor

Veh- Vehicle

β-gal- β -galactosidase

Abstract

G protein-coupled receptors (GPCRs) are the largest family of cell membrane proteins in the human genome. They account for the majority of pharmacological targets due to their physiological diversity and implication in diseases. Despite many efforts to identify the ligands of GPCRs, there are many with no known endogenous ligand, deemed orphans. GPCRs are known to bind a wide variety of ligands, including L-amino acids. This study aimed to investigate the amino acid activation of 72 orphan receptors using a high-throughput screening (HTS) method. The signaling of six orphan receptors was significantly modulated following treatments with a 4/8X mixture of essential/non-essential amino acids: GPR12, GPR26, GPR37L1, GPR84, GPR88, and MRGPRX2. All receptors were activated by the 4/8X mixture except GPR37L1, which, following treatments with individual amino acids, its decrease in signaling was attributed to L-Arg with a fold change of 0.70. The remaining receptors were all significantly activated by L-Phe. In the case of GPR12, GPR84, and GPR88, activation by L-Phe surpassed that of the 4/8X amino acid mixture with fold changes of 2.66, 2.56, and 5.35, respectively, suggesting possible competitive inhibition during the 4/8X treatment. These results suggest that, through activation of GPCRs, L-Phe may play a much more significant role in metabolic processes than once thought. Determination of the biological relevance of this signaling may one day present a great therapeutic potential for future drug discovery.

1. Introduction

1.1 Signal transduction

Cells are required to adapt to the changing conditions of their extracellular environment to survive over time. Adaptation is accomplished through communications with the environment, allowing cells to detect a stimulus and process this information (Sorkin and von Zastrow 2002). Cells are then able to produce a response, thus facilitating adaptation and the coordination of death, growth, metabolism, and proliferation (Sorkin and von Zastrow 2002). These communications are carried out by large signaling networks formed of many pathways. Signaling pathways are dynamic systems that coordinate a diversity of inputs and outputs using four interlinked components: signal molecules, receptor proteins, intracellular signaling proteins and small mediators, and target proteins (Frank 2013).

Signal molecules may range from lipids, peptides, ions, photons, to amino acids to mediate receptor activation at orthosteric or allosteric binding sites (Rosenbaum et al. 2009). Orthosteric binding sites are targeted by endogenous signaling molecules, which directly affect the signal transduction pathway downstream of the receptor (Jensen and Spalding 2004). Allosteric modulators bind at topographically distinct regions from the orthosteric site inducing a change in receptor conformation to alter receptor activity (Wootten et al. 2013). Ligands may fully or partially stabilize the activated conformation of the receptor (agonist), prevent agonist-receptor interactions (antagonists), or yield responses opposing the corresponding agonist (inverse agonists) (Wootten et al. 2013). Antagonists do not inherently cause pharmacological actions through receptors; however, inverse agonists may be useful in the characterization of constitutively active receptors as the binding of the ligand favors a transition to the resting state of the receptor (Jensen and Spalding 2004).

In extracellular signaling, the signal molecule is synthesized and released by the signaling cell before being transported to the target cell, which expresses the receptor protein of interest at its cellular surface (Müller and Schier 2011). Receptors may also be located intracellularly and typically bind small hydrophobic hormones such as steroid hormones and dissolved gases that diffuse across the plasma membrane (Guiochon-Mantel et al. 1996). Cell surface receptors are integral membrane proteins embedded in the phospholipid bilayer of the membrane (Jensen and Spalding 2004). As a result, most cell surface receptors contain domains of hydrophobic intermembrane residues. These receptors must distinguish the unique chemical features of extracellular signal molecules, including molecular size, shape, chirality, and charge to bind, which cannot cross the membrane due to their size or hydrophilicity (Jensen and Spalding 2004). Ligands may also compete for the same binding site leading to competitive activation or inhibition of the receptor (Christopoulos 2002). These factors ultimately determine if and with what affinity signaling molecules will modulate specific receptors. Ligand-bound receptors undergo a conformational change to become activated and bind effector proteins intracellularly beginning a chain of intracellular reactions (Wooten et al. 2013). The reactions making up signal transduction pathways aim to amplify and propagate the signal downstream of the receptor to produce cellular responses, often reaching the nucleus of a cell to target transcription factors and effectively regulate gene expression (Reiter et al. 2012).

1.2 Cell-surface receptors

Cell-surface receptors have been major targets for therapeutic treatments ever since their discovery (Reiter et al. 2012). It was then proposed that the extracellular signal may be antagonized or structurally and chemically imitated by pharmaceutical agents leading to the elucidation of several drugs, mostly targeting cell surface receptors (Christopoulos 2002). Extracellular signaling

molecules are often present in low concentrations; therefore, mechanisms spatially and temporally coordinate the distribution of ligands, which also have a high binding affinity to their specific receptors (Müller and Schier 2011). There are three classes of cell surface receptors, ion-channel-coupled receptors, G protein-coupled receptors (GPCRs), and enzyme-coupled receptors (Jensen and Spalding 2004). Approximately 35% of all currently approved drugs bind to GPCRs, of which only a select number of the superfamily are targeted, demonstrating the great potential of GPCRs in the discovery of novel drug treatments (Sriram and Insel 2018). Thus, researchers' efforts have been focused on characterizing the druggable GPCR-ome as these may present novel therapeutic targets.

1.3 G protein-coupled receptors

GPCRs are the largest family of cell surface receptors in the human genome. They may be classified by their structural similarities: rhodopsin (family A), secretin (family B), glutamate (family C), adhesion and frizzled/taste2, or by their ligand-binding sites as noted by the International Union of Basic and Clinical Pharmacology (IUPHAR) (Husted et al., 2017; Rosenbaum et al., 2009). The rhodopsin family is known to be the largest and most diverse family, accounting for over 80% of human GPCRs of which include olfactory and non-olfactory subcategories (Hanlon and Andrew 2015).

GPCRs, also known as seven-transmembrane (7TM) receptors are characterized by the presence of seven serpentine-like transmembrane α -helices with an extracellular N-terminus and intracellular C-terminus (Wesley et al., 2003). The crystal structure of rhodopsin revealed by Palczewski et al. (2000) illustrated the unique structure of these receptors and the role they play in a diverse range of complex signaling pathways. These receptors undergo ligand-induced conformational shifts to act as a guanine-nucleotide exchange factor (GEF) for heterotrimeric G

proteins, containing α , β , and γ subunits (Sorkin and von Zastrow 2002). The $G\alpha$ subunit exchanges GDP for GTP leading to the activation and separation of the heterotrimeric proteins where the α and $\beta\gamma$ subunits of the G protein may modulate different downstream effector proteins to elicit metabolic responses (Husted et al., 2017). Regulators of G protein signaling (RGS) proteins stimulate the GTPase activity of the $G\alpha$ subunit and the desensitization of the receptor (Dohlman and Thorner 1997). This conversion of the $G\alpha$ subunit promotes the reassociation of the heterotrimeric G protein (Sorkin and von Zastrow 2002). Certain GPCRs interact directly with specific domains of non-G protein effectors, for example, PDZ domains; however, these protein-protein interactions are rare (Reiter et al. 2012).

The attenuation of GPCR signaling is comprised of three highly conserved and regulated processes, receptor desensitization, sequestration, and downregulation (Luttrell and Lefkowitz 2002). Most ligand-bound receptors are phosphorylated almost immediately on serine and threonine residues of the third intracellular loop by G protein-coupled receptor kinases (GRKs), to initiate desensitization through β -arrestin recruitment (Luttrell and Lefkowitz 2002). β -arrestin acts as a multifunctional adaptor and scaffold protein (Luttrell and Lefkowitz 2002). These proteins facilitate the uncoupling of the receptors from the G protein and coordinate the internalization of the receptor through clathrin-dependent endocytic machinery, including clathrin, β 2-adaptin and *N*-ethylmaleimide-sensitive fusion protein (NSF) (Luttrell and Lefkowitz 2002). Clathrin-coated vesicles were originally thought only to modulate endocytosed GPCR activity at the cell surface and were responsible for dephosphorylating and recycling receptors back to the plasma membrane, or targeting them for lysosomal degradation (Gaborik and Hunyady 2004). However, GPCRs located on the surface of intracellular compartments may activate specific signal

transduction pathways, as demonstrated by many growth factor proteins with increased rates of internalization of receptors relative to lysosomal degradation (Sorkin and von Zastrow 2002).

The extent of β -arrestin binding varies between receptors but is most notable between GPCR families. β -arrestin has a relatively low binding affinity for class A GPCRs in comparison to class B receptors (Kim and Caron 2008). Class B receptors, for example, vasopressin type-2 receptor (V_2R), demonstrate prolonged binding as opposed to the transient interaction seen in class A receptors once they have been recruited to clathrin-coated pits (Kim and Caron 2008). This increased stability of the class B GPCR: β -arrestin complex on the surface of the endosomal vesicles is thought to be due to the relatively longer C-terminal tail of the receptors, which increases its number of phosphorylation sites (Kim and Caron 2008).

The unique combinations of ligands, G protein subtypes, cell-type-specific signaling pathways, and regulatory mechanisms increase GPCR functional diversity (Wesley et al., 2003). This diversity, combined with the large family size is, in part, what makes them such important drug targets. Despite extensive research performed on GPCRs in the past few decades, there are currently 91 non-olfactory receptors with no known endogenous or natural ligand, deemed orphan GPCRs (Kroeze et al., 2015). Olfactory receptors detect volatile chemicals but have yet to be identified as targets for drug discovery, unlike non-olfactory GPCRs, which, as a result, are highly investigated by researchers (Roth and Kroeze, 2015). The function of many orphan GPCRs is also unknown, therefore, the deorphanization of these receptors has been a major focus in pharmacology as they present great potential in the development of drug treatments. The discovery that nearly all GPCRs undergo sequestration through β -arrestin binding led to the development of experimental techniques to measure G protein independent β -arrestin recruitment (Lefkowitz

2005). This independence is incredibly useful in the examination of orphan GPCRs whose coupling partners are unknown.

1.4 Tango assay: Experimental strategy to monitor protein interactions

Signal transduction is a rapid and transient response to changes in the extracellular environment. Barnea et al. (2008) developed the Tango assay to elucidate the transient intracellular GPCR: β -arrestin interactions in response to extracellular signals through the quantification of reporter gene activity. The stable luminescent readout allows the quantification of receptor activation for GPCRs, receptor tyrosine kinases (RTKs), and steroid hormone receptors. This novel signaling methodology is conceptually based on the Notch signaling pathway. Protein recruitment by ligand-bound receptors elicits the cleavage of a modified C-terminus tail of the receptor, by a β -arrestin linked with a tobacco etch virus (TEV) protease, and subsequent nuclear translocation leading to the transcriptional activation of a reporter gene (Fig. 1). The receptor is modified to include a TEV protease cleavage site and tetracycline transactivator (tTA) sequence located on its cytoplasmic C-terminus (Barnea et al. 2008). The cleavage site is a seven amino acid sequence specific to the N1a protease of the TEV and is highly specific and effective in monitoring the release, purification, and interactions of proteins. (Barnea et al. 2008; Kroeze et al. 2015). Ligand-induced activation of the receptor will recruit a β -arrestin fused with a TEV protease to the cleavage site. The fusion genes were introduced into cell lines along with tTA-dependent reporter genes. The latter produces a stable and amplifiable readout of the transient signal following the cleavage and irreversible nuclear translocation of tTA (Barnea et al. 2008).

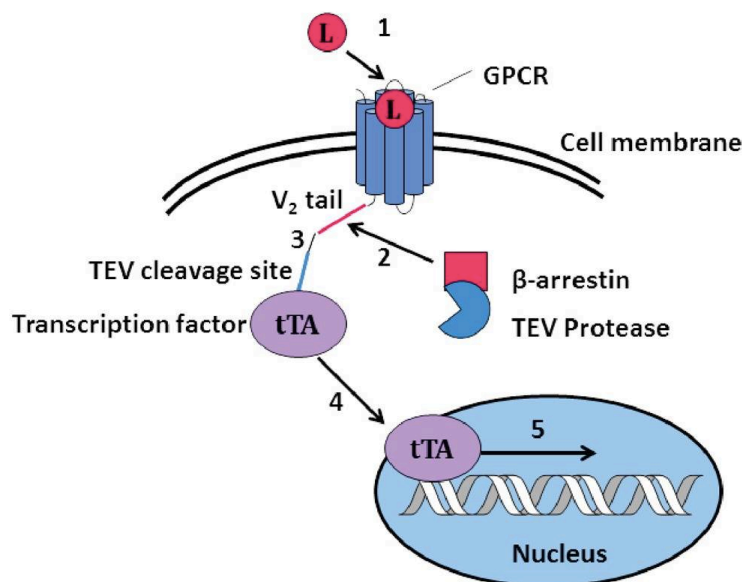


Fig. 1 Schematic of the Tango assay. The general methodology of the ligand-induced β -arrestin recruitment resulting in the nuclear translocation of tTA and activation of luciferase reporter gene transcription. Image adapted from Kroeze et al. (2015).

The advantages of the Tango assay include the independence of G protein coupling, high signal-to-background ratios, and large stable readout signals (Barnea et al. 2008). This assay is a sensitive approach to determine G protein independent β -arrestin recruitment of a receptor, making it a suitable assay for orphan GPCRs (Barnea et al. 2008). The use of the Tango plasmid containing the transcription factor eliminates possible interference from endogenous receptor signaling (Hanson et al. 2009). Independence of the luciferase readout from receptor internalization also allows the interrogation of both slow and quick recycling GPCRs (Hanson et al. 2009). However, despite the many advantages of this assay, it is technologically and economically disadvantageous when performing high-throughput screening (HTS) due to the diversity of signaling pathways, which is a necessary approach when examining the entire druggable GPCR-ome. Thus, the Parallel Receptor-ome Expression and Screening *via* Transcriptional Output-Tango (PRESTO-Tango) method was developed by Kroeze et al. (2015).

1.5 PRESTO-Tango luciferase assay: Investigation of the druggable GPCR-ome

The PRESTO-Tango assay was inspired by the advantages of high content screening (HCS) and bioluminescence resonance energy transfer (BRET), which have previously been used to quantify GPCR: β -arrestin interactions (Kroeze et al. 2015). This method was validated for a parallel and simultaneous interrogation of the druggable GPCR-ome, allowing efficacious screening of biologically active compounds (Kroeze et al. 2015). Readout signals remain independent from G protein coupling making this a suitable assay for the investigation of orphan GPCRs and a promising approach to the potential identification of both synthetic and naturally occurring agonists of these orphan GPCRs.

The original Tango plasmid constructs for each GPCR were modified and are easily accessible by the scientific community (Fig. 2). The PRESTO-Tango plasmids include restriction enzyme cut sites intended to develop a modular design that may be altered for specification to various studies (Kroeze et al. 2015). At the 5'-end, there is an HA signal to promote membrane localization of receptors, as well as a FLAG epitope tag to monitor cell surface expression of receptors through immunohistochemistry. The sequences of a cleavage site for the N1a protease from TEV and the tTA protein are located at the 3'-end of the Tango-ized receptor. A β -arrestin2:TEV fusion gene is stably expressed in the HTLA cell line used in this assay to facilitate cleavage. HTLA cells are a modified human embryonic kidney 293 (HEK293) cell line stably expressing a tTA-dependent luciferase reporter gene and a β -arrestin2:TEV fusion gene (Kroeze et al. 2015). Interposed between the GPCR coding sequences (CDS) and TEV cleavage site, the sequence from the C-terminus of the V₂ vasopressin receptor (V₂ tail) promotes β -arrestin recruitment due to its robust interaction with β -arrestin2 (Barnea et al. 2008; Hanson et al. 2009;

Vrecl et al. 2009). The most prevalent form of the GPCR CDS was codon-optimized for use in human cell lines.



Fig. 2 PRESTO-Tango plasmid construct. The G protein-coupled receptor coding sequence (GPCR CDS) is flanked by the HA signal and flag epitope at the 5'-end of the plasmid, and the V₂ tail, tobacco etch virus (TEV) cleavage site and the tetracycline transactivator (tTA) protein at the 3'-end to form the PRESTO-Tango plasmid to be transfected into HTLA cells. Blue and green arrowheads indicate enzyme restriction sites for Cla I and Age I, respectively. Image adapted from Kroeze et al. (2015).

1.6 Activation of GPCRs

Small organic molecules may bind within the hydrophobic core of GPCRs, while most native agonists bind to the extracellular loops that join TM domains or the amino terminus of the receptor (Kobilka 2007). This makes sense, given that these regions are the most structurally diverse of GPCRs giving rise to the diverse range of functional differences between receptors (Kobilka 2007). Historically, receptor function has been classified as a two-state model. The ligand-induced conformation changes cause the receptor to become activated and elicit a response or stabilized in the inactive state (Violin et al. 2014). This simplistic view has since been contradicted by reports of compounds with varying relative assay-dependent potencies for a specific receptor (Spongier et al. 1993; Watson et al. 2000). The conformation of ligand-bound receptors is now described as a spectrum where each ligand-stabilized structure, when compared to an unbiased endogenous agonist, induces qualitatively and quantitatively varying pharmacological responses, this is known as biased signaling (Violin et al. 2014). Biased ligands bind to a subset of receptor conformations that favor certain effector proteins and elicit distinct signaling pathways (Wootten et al. 2013). Increased specificity and precision of the agonist

decreases the occurrence of off-target signaling, which may have led to adverse effects, raising the prospect of safer and more efficacious drug treatments (Violin et al. 2014).

To characterize receptor activity in response to particular ligands, Furchgott (1964) developed dose-response curves (DRCs) from receptor mechanism theories and described many pharmacological concepts, including affinity and efficacy. The efficacy of a ligand is the maximum response it can produce, while affinity describes the strength of the intermolecular force between receptor and ligand. The potency is the concentration necessary to achieve half of a maximal receptor response and is typically in comparison to a reference ligand (Reiter et al. 2012). Therefore, two compounds may be equipotent yet bind receptors with different affinities, which are then represented by differing efficacies (Reiter et al. 2012).

The diverse range of ligands selective for GPCRs continues to increase as receptors are deorphanized. What has become increasingly clear to researchers through the deorphanization process over the past two decades is the importance of metabolites not only as energy sources in metabolic pathways but as extracellular signaling molecules, most notably in the gastrointestinal (GI) tract. Together these discoveries suggest that GPCRs account for most of the nutrient-sensing occurring in the GI tract (Wootten et al., 2013). Nutrient metabolites are known to function similarly to neurotransmitters and hormones; however, they tend to target receptors expressed on the cells that produce them or on neighboring cells (i.e. through autocrine or paracrine signaling, respectively) (Husted et al. 2017). Metabolite receptors are highly expressed and regulated in endocrine and metabolic organs, often regulating the secretion of hormones (Engelstoff and Schwartz 2016). Expressed similarly in the adaptive and innate immune system, metabolite receptors, for example, free fatty acid receptors, GPR109A, and GPR35, are abundant in immune cells and generally derive beneficial anti-inflammatory effects (Alvarez-Curto and Milligan 2016;

Thorburn et al. 2014). Many receptors, including metabolite receptors, have also been reported to bind small allosteric modulators through binding within the transmembrane segments in addition to their native agonists as in the case of the calcium-sensing receptor (CaSR) (Conigrave et al. 2000; Kobilka 2000, 2007; Wootten et al. 2013).

1.7 Allosteric modulators in drug discovery

Classic drug design focuses on the binding of the orthosteric site of an enzyme or receptor (Abdel-Magid 2015). Despite being highly effective, this approach often has many adverse side effects. The high sequence homology of orthosteric sites is problematic when targeting receptors due to off-target signaling, and these drugs may act as complete activators or inhibitors, thus not allowing a spectrum of modulation (Abdel-Magid 2015). Among subfamilies of GPCRs, allosteric sites are typically less conserved than the primary active sites as there is decreased evolutionary pressure to accommodate the binding of the endogenous ligand (Christopoulos 2002). Other disadvantages of using orthosteric binding sites clinically are the consequent desensitization and up/downregulation of the receptor leading to decreased drug efficacy over time (Wild et al. 2014). Allosteric ligands may act as intrinsic agonists, positive allosteric modulators (PAMs), negative allosteric modulators (NAMs), or neutral allosteric ligands (NALs, previously termed silent allosteric modulators) (Wootten et al. 2013). The diversity of allosteric binding sites allows for increased target selectivity for subtypes of receptors minimizing the chance of off-target signaling (Wild et al. 2014).

Allosteric ligands may lack intrinsic activity and may be structurally modified to fine-tune their affinity and efficacy for a specific receptor (Conn et al. 2009). Ligand-specific conformational changes in the GPCR due to allosteric binding induces distinct signaling pathways exhibiting ligand bias (Jensen and Spalding 2004). The effects of allosteric modulators that lack

intrinsic activity are dependent on the presence of the orthosteric ligand; therefore, these maintain the spatial and temporal aspects of the physiological signal and thus protect against potential drug overdose (Wootten et al. 2013). Allosteric signaling is an emerging pharmacological tool that presents many distinct advantages over orthosteric signaling, has identified a variety of currently marketed drugs, and will continue to provide valuable clinical benefits.

While this is a relatively new approach to drug discovery, there are allosteric modulator drugs for the treatment of various disorders. In the treatment of Parkinson's disease and schizophrenia, PAMs target dopamine 1 receptor (D1), which may also carry benefits for patients with Alzheimer's disease, depression and attention deficit-hyperactivity disorder (ADHD) (Abdel-Magid 2015). Additionally, metabotropic glutamate receptor subtype 2 (mGluR2), which belongs to a family of class C GPCRs activated by glutamate, also binds NAMs in the treatment of central nervous system (CNS) disorders (Abdel-Magid 2015). Other GPCRs that show potential in drug therapy through allosteric modulators include CaSR. Extracellular Ca^{2+} ions primarily regulate the activation of CaSR; although, it has also been shown to bind L-amino acids, oligopeptides, and polyamines with a lower affinity (Conigrave et al. 2000; Rogers et al. 2015). GPR35 is a promiscuous receptor detecting many aromatic acids, and synthetic agonists with high potency (MacKenzie et al. 2014). Some focus has been shifted to amino acids and their derivatives as they are considered important ligand metabolites in GPCR signaling since being reported as agonists selective for GPR142, GPR35, FPR1-2, CaSR, GPRC6A, and T1R1/ T1R3 (Husted et al. 2017).

1.8 Amino acid sensing receptors

Amino acid sensing receptors link the changes in protein content of the body and amino acid metabolism with metabolic responses and are involved in the regulation of appetite and energy balance (Liu et al. 2016). Ingested protein must coordinate the activity of both the peptide

transporter 1 (PEPT1), which transports amino acids in a proton-dependent manner from the intestinal lumen into enterocytes, and CaSR, which senses amino acids (Diakogiannaki et al. 2013). Both are expressed in enterocytes and at the basolateral membrane of enteroendocrine cells suggesting that amino acid sensing receptors may be expressed in these cells as well. This is the case for GPR142, which is located in enteroendocrine cells and recognizes the essential amino acids L-Tryptophan (L-Trp) and L-Phenylalanine (L-Phe) (Lin et al. 2016).

Most amino acid receptors are selective for the L-configuration of amino acids; yet, GPR109B (HM74) is known to bind D-phenylalanine, D-tryptophan, and D-kynurenine, a metabolite of the latter (Irukayama-Tomobe et al. 2009). The predominant binding of the L-isomer makes sense, given that this is the predominant configuration of naturally occurring amino acids in the human body and most other living organisms (Armstrong et al. 1993).

Many class C GPCRs, including the Taste Receptor Type 1 (T1R) family, CaSR, and GPRC6A, are major amino acid sensing receptors. The T1R1/T1R3 heterodimer is located throughout the gastrointestinal (GI) tract and is highly selective for most L-amino acids except for the non-polar aromatic amino acid, tryptophan (Liu et al. 2016). In addition to Ca^{2+} ions, CaSR mediates cholecystokinin (CCK) secretion through activation by L-amino acids most notably, L-Phe and L-Trp, and small peptides (Conigrave et al. 2000; Liu et al. 2016). Ca^{2+} also enhances the signaling of GPRC6A. GPRC6A shares high structural homology with CaSR, and its activity is regulated through binding, both orthosterically and allosterically, of a mixture of basic L-amino acids and cations (Pi and Quarles 2012).

1.9 Summary

Based on the above information, this study aimed to investigate the amino acid activation of orphan GPCRs. This was accomplished through a parallel and simultaneous comparison of

changes in luminescent signaling of receptors treated with a mixture of amino acids using an HTS methodology of the PRESTO-Tango assay. These findings led to the identification of the amino acid modulating individual receptor activity. This data has important implications in the characterization of the druggable GPCR-ome and may provide valuable insights into drug development selective for newly orphanized receptors.

2. Methods

2.1 Cell culture: Modified human embryonic kidney cells

HTLA cells (a human embryonic kidney 293 (HEK293) derived cell-line stably expressing tTA-dependent luciferase reporter gene and a β -arrestin2-TEV fusion gene) were grown in high glucose (4.5 g/L) complete media (Dulbecco's Modification Eagle's Medium 1X Mod. (DMEM; Wisent Bioproducts, 319-005-CL), supplemented with 10% fetal bovine serum (FBS; Wisent BioShop, 080105), 1% penicillin-streptomycin (P/S), 100 μ g/mL hygromycin (BioShop, HYG002.1), and 2 μ g/mL puromycin (BioShop, PUR333.100). Media was changed every two days, and cells were sub-cultured when 70-80% confluency was reached. To sub-culture, media was removed, and cells were washed with 1X Phosphate Buffered Saline (PBS). Cells were trypsinized with 0.25% trypsin/2.21 mM ethylenediaminetetraacetic acid (EDTA) (Wisent Bioproducts, 325-043-EL), and incubated for 2 min at 37°C. Lifted cells were mixed with high glucose complete media, centrifuged, and resuspended in high glucose complete media. Cell suspension was split at a ratio of 1:20.

2.2 DNA minipreps

Escherichia coli (*E. coli*) cultures of orphan receptors were propagated on prewarmed LB ampicillin (AMP; 50 μ g/mL) (Fisher Scientific, BP1760-5) plates from a PRESTO-Tango GPCR

library (Roth Lab, Addgene Kit #1000000068), and incubated at 37°C to grow overnight. The plasmids, pBSK, and pCMV- β -galactosidase (β -gal), were also propagated from frozen stocks. Single colonies were selected to grow in liquid LB medium (Fisher Scientific, BP1426) with 50 μ g/mL AMP, and incubated shaking overnight at 37°C. The plasmid vector DNA was prepared using the plasmid miniprep system (Machery Nagel, 740490.50), following the manufacturer's protocol. DNA was quantified using nanodrop and purity was verified through agarose gel electrophoresis. A sample of *E. coli* culture was stored in 50% glycerol at -80°C, and a sample of eluted DNA was stored in tris(hydroxymethyl)amino-methane (TRIS)-EDTA buffer (pH 8.0) at -20°C for long-term storage.

2.3 MTT assay of cell viability

To ensure cells would be viable following amino acid treatments, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay (MTT assay) was performed. Cells were grown, transfected with expression vectors encoding the receptor GPR142 and green fluorescent protein (GFP), and treated as described for the PRESTO-Tango luciferase assay protocol. Treatments included 1X essential amino acids (Wisent, 321-010-EL), 1X non-essential amino acids (Sigma, M7145), and 1/1X, 4/8X and 25/50X essential/non-essential amino acid mixtures (See Appendix 1 and 2 for individual amino acid concentrations). On day 4, GFP fluorescence was viewed by microscopy (Thermofisher EVOS-FL) to assess transfection efficiency, and cells were incubated in 1 mg/mL of thiazoyl blue tetrazolium bromide (MTT) for 2 hrs at 37°C. The media was removed, and the formazan product was solubilized in 100 μ L dimethyl sulfoxide (DMSO). Cells were protected from light and shaken for 15 min prior to absorbance measurement at 570 nm.

2.4 PRESTO-Tango luciferase assay

HTLA cells were plated at 25,000 cells/well in DMEM containing 1% FBS and incubated overnight at 37°C with 5% CO₂ (day 1). The transfection mixture containing: 25 ng of β -gal, 50 ng pBSK, 25 ng of PRESTO-Tango receptor plasmid, 4 μ g/mL polyethylenimine (PEI) and 1X Opti-MEM reduced serum medium with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.4 g/L sodium bicarbonate and *L*-glutamine (Opti-MEM; Gibco, 31985-070) was incubated for 15 min prior to transfection of cells in 30% of original media volume (day 2). A trial with GFP in lieu of the receptor plasmid was used to examine transfection efficiency visually by microscopy. To verify the efficacy of the β -gal expression vector, receptor DNA was replaced with pBSK, a non-coding DNA vector. The GPCR LPAR1 was used as a positive control for receptor activation. Transfected cells were incubated overnight at 37°C with 5% CO₂. On day 3, orphan GPCRs were treated with 3X Opti-MEM (vehicle) and a 4/8X mixture of essential/non-essential amino acids for the high throughput screen then incubated overnight at 37°C with 5% CO₂. GFP and pBSK trials underwent vehicle treatment, LPAR1 was treated with Opti-MEM containing a final concentration of 10% FBS. The following day, media was removed, cells were lysed in 1X Reporter Lysis Buffer (Biotium, 99821), incubated for 15 min while shaking, and frozen at -80°C to aid with lysis (day 4). To measure β -gal activity, thawed lysate was combined with 1X β -gal assay buffer (0.1 M sodium phosphate buffer (pH 7.3), 1 mM MgCl₂, 0.067% (w/v) ortho-nitrophenyl- β -D-galactopyranoside (OPNG), 50 mM-mercaptoethanol), incubated for 5 min, and absorbance was read at 420 nm for the β -gal assay. The luciferase assay was performed on lysate in 0.25 mg/mL D-luciferin stock in assay buffer (Biotium, 30028-L2), and incubated for 5 min while shaking, prior to luminescence being read at 560 nm using a Synergy HT microplate reader. The PRESTO-Tango assay was repeated with receptors that had a significant change in

luminescent signaling during the HTS. Receptors were treated with the 4X essential amino acids and 8X non-essential amino acid mixtures separately to determine which category of amino acids were altering receptor activity. The same procedure was used for treatments with the individual amino acids of the activating category. Concentrations of individual amino acids were diluted in Opti-MEM to be equivalent to those found in the original 4/8X treatment mixture. All trials were performed in technical and biological triplicates.

2.5 Statistical analyses

Luminescence was standardized against the relative absorbance of the β -gal assay to calculate the relative luminescence (RLU). Fold change was expressed relative to the vehicle-treated cells. RLU and fold change were analyzed using R and Prism (GraphPad Software). In R, the Shapiro-Wilks test was used for normal distribution, Bartlett's test for homogeneity of variances, two-tailed t-test to check for a significant difference, and Wilcox test to evaluate significance for cases where assumptions for normalcy were not met. A one-way analysis of variance (ANOVA) with multiple comparisons comparing the mean of each column with the mean of a control column, vehicle (Veh) or the non-essential (NE) amino acid mixture where indicated, was performed, with $p < 0.05$ vs. Veh or Veh+NE. All data are expressed as mean \pm standard error of the mean (SEM).

3. Results

3.1 Concentrated amino acids decrease cell viability

In elevated concentrations, amino acids may become toxic to cells. To ensure cell viability following treatments during the PRESTO-Tango HTS an MTT assay was performed on HTLA cells transfected with GPR142, a known amino acid sensing GPCR, and treated with varying

concentrations of the essential and non-essential amino acid mixtures (Fig. 3) (Lin et al. 2016). Absorbance was measured at 570 nm and expressed as fold change relative to vehicle (Veh). Treatments increasing in amino acid concentration had increased viability of HTLA cells, reaching optimal viability with the 4/8X essential/non-essential amino acid mixture, following which treatments became toxic to cells. The 4/8X mixture induced a significant increase in cell viability, and concentrations of individual amino acids within the mixture are similar to those found in human plasma (Scriver and Clow 2005). As such, the 4/8X essential/non-essential amino acid mixture was chosen as the treatment for the HTS.

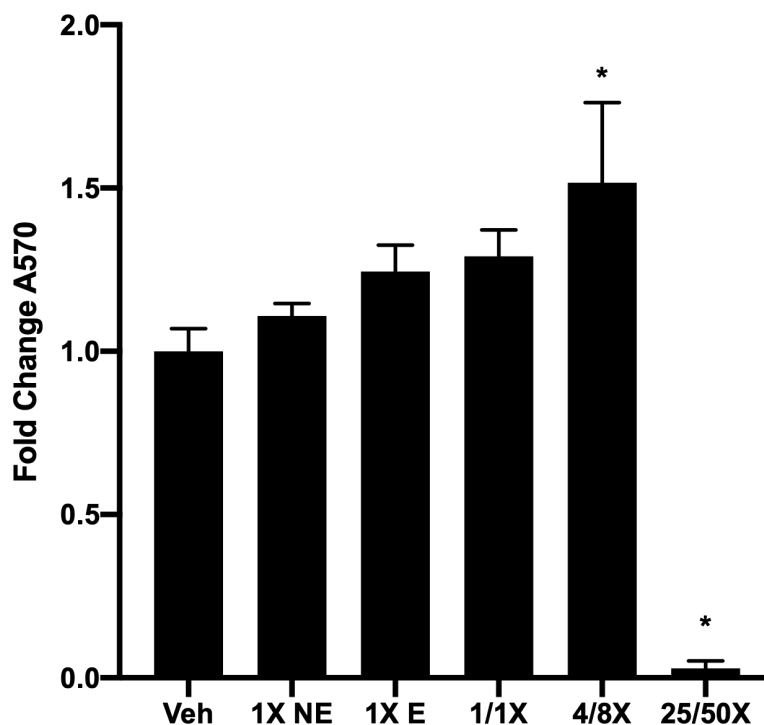


Fig. 3 Effect of treatments with varying concentrations of amino acids on cell viability. An MTT assay was performed on HTLA cells transfected with GPR142 to determine the effect of treatments with non-essential (NE) and essential (E) amino acids on cell viability relative to vehicle (Veh) treatment. Treatments included: Veh, 1X NE, 1X E, and 1/1X, 4/8X and 25/50X E/NE amino acid mixture. A570 was determined and expressed as fold change relative to Veh. * $p < 0.05$ vs. Veh. Data are shown as mean \pm SEM.

3.2 Amino acids significantly modulate orphan GPCR activity

Amino acids have proven useful not only as fuel in metabolic pathways but as extracellular ligands binding several GPCRs (Husted et al. 2017). To investigate the activation of orphan GPCRs in response to treatments of amino acids, an HTS method of the PRESTO-Tango assay was performed on HTLA cells transfected with 72 orphan receptors and treated with a 4/8X essential/non-essential amino acid mixture (Fig. 4). Luciferase/ β -galactosidase activity (RLU) was determined and expressed relative to vehicle treatment. This change in luminescent signaling (fold change RLU) is representative of a shift in receptor activity. The significance (p-value) of the fold change RLU of receptors across the three biological replicates is represented using a heat map (Fig. 4A, see Appendix 3 for data). Six orphan receptors (highlighted in red): GPR12, GPR26, GPR37L1, GPR84, GPR88, and MRGPRX2 showed a significant change in luminescent signaling when compared with untreated controls. To determine whether this change represented an activation of receptors or a decrease in the activity of constitutively active receptors, the fold change RLU of the six orphan receptor candidates was represented graphically (Fig. 4B). All receptors had increased luminescent signaling in response to amino acid treatment when compared to vehicle except GPR37L1, which had a fold change of 0.50. GPR88 and MRGPRX2 had the greatest fold change activation of 3.36 and 3.16, respectively, when treated with the 4/8X mixture of amino acids.

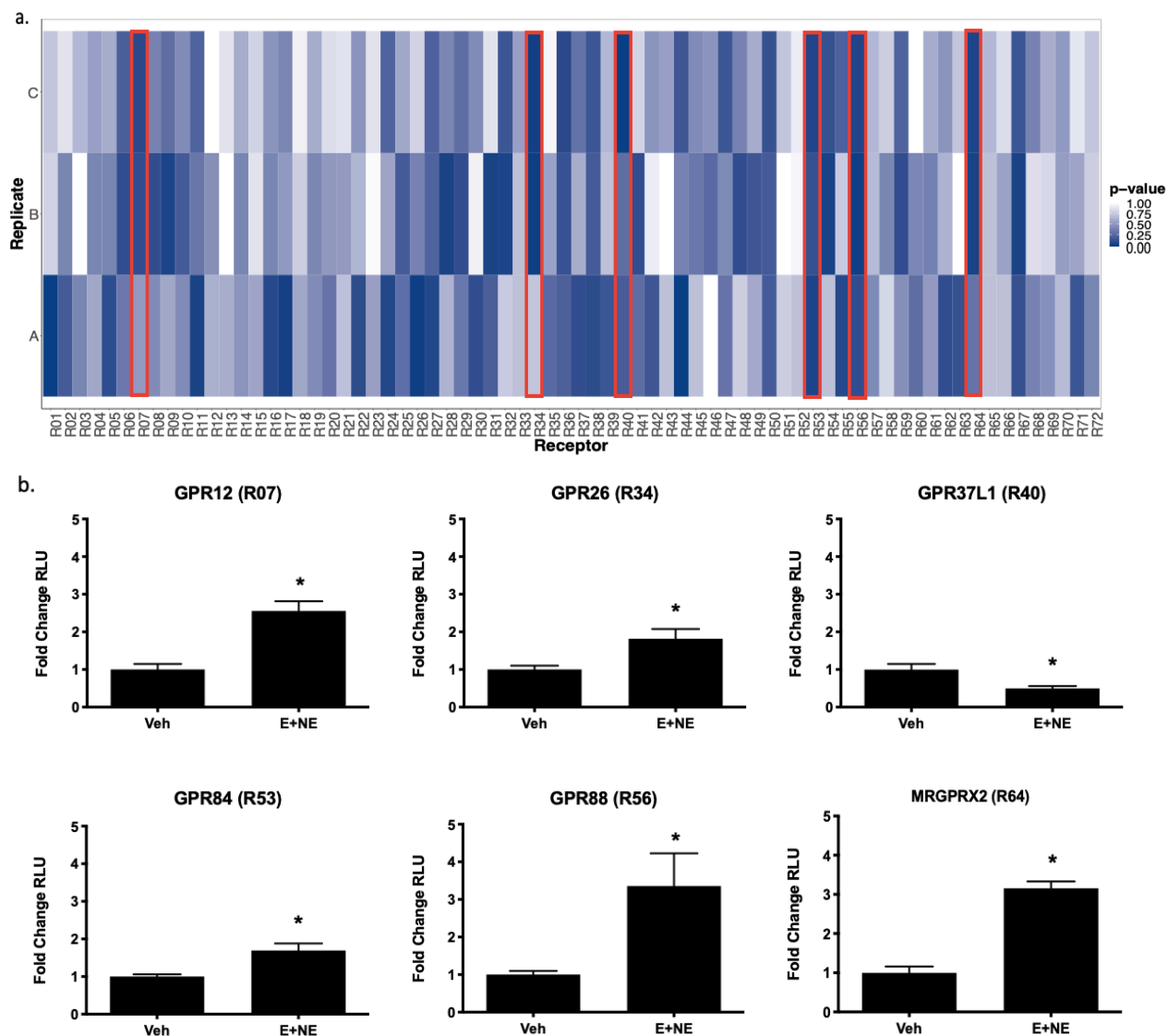


Fig. 4 Orphan receptor candidates activated by a mixture of amino acids. To determine if orphan GPCRs would have significantly altered function in response to treatments of amino acids, a PRESTO-Tango assay was performed on HTLA cells transfected with Tango-ized orphan GPCRs and treated with vehicle (Veh), and a 4/8X essential/non-essential amino acid (E+NE) treatment. **(a)** A heat map of amino acid-treated orphan receptors. The significance level (p-value) of the change in luminescent signaling between amino acid treatments and control for the 72 orphan receptors. The three biological replicates are denoted as A, B, and C. GPR12, (R07), GPR26 (R34), GPR37L1 (R40), GPR84 (R53), GPR88 (R56), and MRGPRX2 (R64) had significant changes in luminescent signaling and are highlighted in red. **(b)** Luciferase/ β -galactosidase activity (RLU) was determined for the six orphan receptor candidates and expressed relative to Veh as fold change. * $p < 0.05$ vs. Veh. Data are shown as mean \pm SEM.

3.3 Orphan GPCRs may be activated by essential or non-essential amino acids

To determine which subgroup of amino acids were significantly altering the activity of receptors, the above experiment was repeated with the six orphan receptor candidates treated with vehicle, the 4/8X essential/non-essential amino acid mixture (E+NE) to confirm changes in luminescent signaling, and the essential (E) and non-essential (NE) amino acid mixtures separately. In a preliminary experiment, receptors seemed to respond to the essential amino acid mixture which led to treatment of receptors with the individual essential amino acids. This included replicates of the 4/8X mixture and the essential and non-essential mixtures separately to confirm the results of the HTS and preliminary experiment, respectively. Following the completion of the three biological replicates, treatments of the essential and non-essential mixtures independently did not yield a significant change in receptor luminescent signaling relative to vehicle treatment nor each other, except in the case of GPR37L1 (Fig. 5). GPR37L1 had a significant decrease in activity relative to the vehicle and the non-essential mixture when treated with the essential amino acid mixture. Treatments with the mixtures separately did not alter receptor activity as effectively as the 4/8X mixture. However, amino acid-mediated activation of receptors did show a slightly increasing trend with the essential mixture, although this ultimately was not significant.

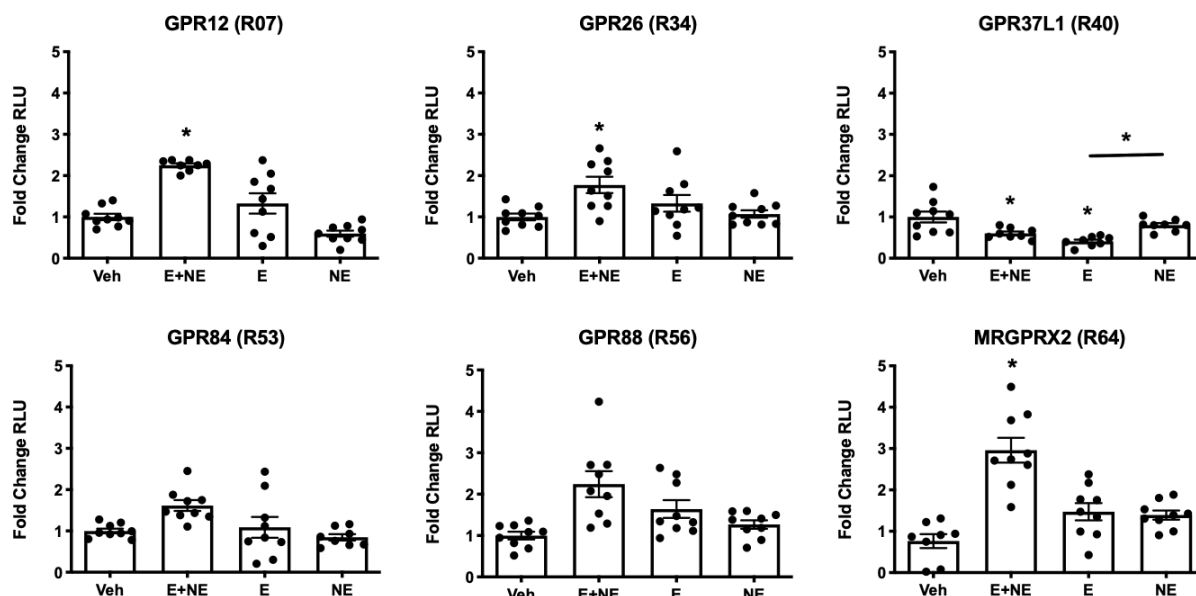


Fig. 5 Amino acid-dependent signaling of orphan GPCRs. To determine which amino acid group was affecting the Tango-ized orphan GPCRs, HTLA cells were transfected and treated as indicated with vehicle (Veh), and the 4/8X essential/non-essential (E+NE), 4X essential (E) and 8X non-essential (NE) amino acid mixtures separately. Luciferase/ β -galactosidase activity (RLU) was determined for the six orphan receptor candidates and expressed relative to Veh as fold change. * $p < 0.05$ vs. Veh + NE. Data are shown as mean \pm SEM.

3.4 L-Phenylalanine significantly activates many class A orphan GPCRs

The PRESTO-Tango assay was performed on HTLA cells transfected with the six orphan receptor candidates and treated with the individual amino acids comprising the essential amino acid mixture to investigate which amino acid was altering receptor activity (Appendix 4). The decreased luminescent signaling of GPR37L1 was greatest when treated with L-Arginine (L-Arg) represented by a fold change of 0.70 relative to vehicle treatment (Fig. 6). Surprisingly, all receptors that were significantly activated by the amino acid mixture (i.e. GPR12, GPR23, GPR84, GPR88, and MRGPRX2) were significantly activated by L-Phenylalanine (L-Phe) when treated with the individual amino acids (Fig. 6). Even GPR37L1, which had decreased activity when treated with the 4/8X mixture, showed activation following L-Phe treatment with a fold change of

1.48. The activation of certain receptors by L-Phe surpassed that of the 4/8X amino acid mixture, as is the case of GPR12, GPR84, and GPR88 with fold changes of 2.66, 2.56, and 5.35, respectively.

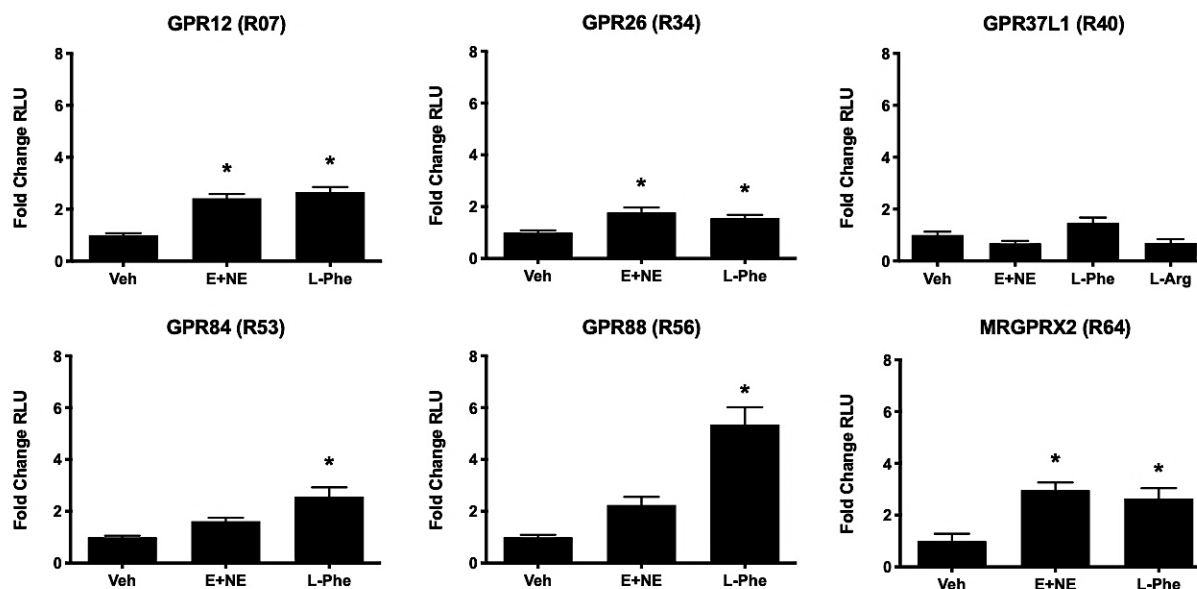


Fig. 6 Identification of individual essential amino acids affecting orphan GPCR signaling. HTLA cells transfected with the indicated Tango-ized orphan GPCR were treated with vehicle (Veh), the 4/8X essential/non-essential (E+NE) amino acid mixture, and the individual amino acids of the essential amino acid mixture at a concentration of 4X (See Appendix 1 and 4). The activity of GPR37L1 was most decreased by L-Arginine (L-Arg), whereas all other orphan receptors involved in this experiment were significantly activated by L-Phenylalanine (L-Phe). Luciferase/ β -galactosidase activity (RLU) was determined and expressed relative to Veh as fold change. * $p < 0.05$ vs. Veh. Data are shown as mean \pm SEM.

4. Discussion

The goal of this study was to determine if the activity of orphan GPCRs could be significantly modulated by L-amino acids. Six of the investigated orphan receptors were significantly modulated following treatments with a mixture of essential and non-essential amino acids that was non-toxic to cells, and where concentrations of individual amino acids are thought to emulate fasting levels in human plasma (Fig. 4A) (Scriver and Clow 2005). All of the orphan receptor candidates were significantly activated by the amino acid mixture except GPR37L1, which had a

decrease in activity (Fig. 4B). It is unclear whether the orphan receptor candidates were activated by the essential or non-essential amino acid mixture. The essential amino acid mixture had a slight trend of greater activation than the non-essential mixture when used as separate treatments; however, this difference was not significant except in the case of GPR37L1 (Fig. 5). The decreased activity of GPR37L1 was attributed to L-Arg with a fold change of 0.70 (Fig. 6). Interestingly, receptors that were activated by the 4/8X mixture were all significantly activated by L-Phe, in some instances with a greater fold change than the mixture itself. GPR37L1 was also activated by L-Phe but to a lesser extent (Fig. 6). Altogether, this study provides evidence that the activity of GPCRs, specifically, class A orphan GPCRs are significantly modulated by L-amino acids and L-Phe may play a larger role in physiological processes via the signaling of these receptors than anticipated.

4.1 Amino acids act as extracellular signaling molecules

Traditionally, amino acids and other metabolites were only considered important in the regulation of energy sources and protein synthesis. However, growing evidence suggests that L-amino acids act as extracellular signaling molecules through the activation of GPCRs, including GPR142, and many class C GPCRs (Husted et al. 2017). Herein we report that the activity of many class A orphan GPCRs is significantly modulated by L-amino acids (Fig. 4). Previously identified amino acid sensing receptors are typically functionally related to digestion and the GI tract or found in immune cells (Husted et al. 2017). Therefore, the receptor candidates identified by this study: GPR12 (R07), GPR26 (R34), GPR37L1 (R40), GPR84 (R53), GPR88 (R56), and MRGPRX2 (R64), may have similar functions to previously described amino acid sensing receptors despite their wide range of expression.

Following a preliminary assay, it appeared that the change in luminescent signaling of all receptors was attributed to the essential amino acid mixture; although, this detail remained elusive following the completion of the biological replicates (Fig. 5). The essential amino acid mixture did have a greater impact than the non-essential amino acid mixture on receptor activity, but this was not significant relative to vehicle treatment nor each other, except for GPR37L1. Amino acids in the non-essential mixture may be altering receptor activity since both mixtures were unable to affect luminescent signaling as efficiently as the 4/8X mixture. Furthermore, many non-essential amino acids have been identified as functional amino acids, meaning these amino acids regulate essential metabolic pathways involved in the maintenance of the development, reproduction, and overall wellbeing of the organism (Wu 2010). Glutamine, glutamate, and proline are known to play a role in nutrient metabolism, gene expression, and oxidative stress (Brasse-Lagnel et al. 2009; Bruhat et al. 2009; Vernone et al. 2019; Wu et al. 2011). Moreover, glutamine and glutamate have been shown to activate the heterodimeric taste receptor complex T1R1/T1R3 functioning in sensing amino acid availability and the regulation of autophagy through the mTOR complex 1 (Wauson et al. 2012; Zheng et al. 2016; Zhou et al. 2016). Future experiments treating receptor candidates with the individual amino acids of the non-essential mixture could help to identify other amino acids activating these orphan receptors, which may provide further explanation as to why there was no significant difference in the signaling of receptors in response to treatments with the essential and non-essential mixtures separately.

4.2 GPR37L1

Of these orphan receptor candidates, all were activated by the treatment except GPR37L1, which had a decrease in luminescent signaling (Fig. 4B). This decrease in the signaling of GPR37L1 was greatest when the receptor was treated with the basic amino acid, L-Arg, suggesting

that this may be acting as a NAM in the amino mixture favoring the resting state of the receptor, an allosteric antagonist which is preventing an agonist in the media from binding to the receptor, or an inverse agonist (Wootten et al. 2013) (Fig. 6). GPR37L1 is known to be a constitutively active receptor; therefore, it is more likely that L-Arg is an inverse agonist of the receptor since these are often useful in the characterization of constitutively active receptors as the binding of the ligand favors a transition to the resting state of the receptor (Jensen and Spalding 2004). However, until further investigation, the other two possibilities for this ligand cannot be discredited.

Characterization of the activity of GPR37L1 remains elusive after many years of research. GPR37L1 is primarily expressed in the nervous system and is particularly enriched in astrocytes (Liu et al. 2018). Signaling of this receptor is thought to inhibit cyclic adenosine monophosphate (cAMP) production and is implicated in glio- and neuroprotection through binding of prosaptide (Meyer et al. 2013). Studies have shown that resting levels of cAMP in astrocytes, where GPR37L1 is particularly abundant, are not anywhere near saturation (Goldman and Chiu 1984; Tardy et al. 1981). This finding is expected given the constitutive activity of GPR37L1; one would expect decreased amounts of cAMP in astrocytes. A study by Meyer et al. (2013) suggested that prosaptide, a peptide derived from prosaposin, stimulated $G\alpha_i$ coupling to GPR37L1 and subsequent phosphorylation of extracellular signal-regulated kinase (ERK). Their findings were later challenged with reports suggesting that this receptor coupled to $G\alpha_s$ proteins and that regulation of its constitutive activity occurred via cleavage of an extracellular domain of the receptor; although, this has since been retracted (Coleman et al. 2016). Liu et al. (2018) were able to support the findings of Meyer et al. (2013) and hypothesize that molecules based around the structure of prosaptide may also regulate neuronal and glial physiology following a nerve injury. Despite there being a possible agonist identified for this receptor, comparatively little is known on

how to decrease its constitutive activity and what implications this may have. We believe this is the first study to show inhibition of GPR37L1 activity and to do so by L-Arg.

Arginine is a common substrate for enzymes and a precursor of nitric oxide (NO) and agmatine which have antiproliferative and antimicrobial effects, both playing a role in the regulation of airway inflammation in asthmatic individuals (Gogoi et al. 2016; Leiss et al. 2014; Satriano 2004). $G\alpha_i$ positively modulates L-Arg as part of a feedback mechanism to induce insulin secretion (Leiss et al. 2014). The positive charge of arginine, among other cationic amino acids, triggers depolarization of pancreatic β -cells, influx of cytosolic Ca^{2+} , and subsequent release of insulin (Henquin 2004). L-Arg-induced insulin release is also modulated through GPCR activation, including the activation of GPRC6A, a promiscuous receptor to basic amino acids (Smajilovic et al. 2013). It is unclear the downstream effect of L-Arg-inhibition of GPR37L1; this may increase cAMP production or regulate another pathway altogether via $G\alpha_i$ proteins. Future studies may be performed to determine the G protein that GPR37L1 couples to following L-Arg binding, examine the effect of L-Arg on cAMP production by way of this receptor, and locate where these interactions may occur *in vivo* to evaluate whether L-Arg could affect the glio- and neuroprotective properties of this receptor.

4.3 L-Phenylalanine activation of GPCRs may play a large role in regulating physiological functions

Surprisingly, the activity of all receptors that had significant amino acid activation during this study was attributed to L-Phe, and even GPR37L1 despite being deactivated by the mixture of amino acids was activated by L-Phe, although this was not significant (Fig. 6). The activation of receptors by L-Phe in the case of GPR12, GPR84, and GPR88 surpassed that of the 4/8X amino acid mixture, suggesting that there may have been competitive inhibition of receptors when treated

with the essential/non-essential mixture (Christopoulos 2002). The occurrence of competitive inhibition makes sense, given that many of the essential amino acids share structural and chemical similarities, and many activated the receptors as well relative to vehicle treatment (Appendix 4).

L-Phe is a neutral essential aromatic amino acid involved in various biochemical pathways, including protein and tyrosine synthesis (Williams et al. 2008). This amino acid has been shown to be a potent activator of CasR to regulate food intake, gut hormone release, and glucose tolerance; adhesion receptors ADGRG1 and ADGRG3 found in immune cells; and GPR142 which is involved in glucose metabolism (Alamshah et al. 2017; Chen et al. 2019; Hsiao et al. 2018; Lin et al. 2016). The effects of L-Phe are widespread throughout the body. Increased levels of this amino acid may be caused by phenylketonuria (PKU), an inherited disorder characterized by a mutation in phenylalanine hydroxylase (PAH), which may lead to impaired cognitive abilities (Oates et al. 1963; Williams et al. 2008). However, L-Phe has also been used therapeutically for patients with depression, ADHD, and osteoarthritis, since it is a precursor for norepinephrine and dopamine; as well as in Parkinson's patients to produce L-dihydroxyphenylalanine (L-DOPA) *via* tyrosine; and in patients with vitiligo as a precursor of melanin (Gianfaldoni et al. 2018; Hsu et al. 2014; Slominski et al. 2012). The activation of multiple GPCRs in this study by L-Phe suggests that these receptors may also play a role in regulating the physiological functions of L-Phe.

4.4 GPR12

Class A GPCRs are the largest and most diverse subfamily of GPCRs, including both olfactory and non-olfactory receptors (Zhou et al. 2019). GPR12 belongs to a structurally related (57-61% sequence identity) family of constitutively active receptors consisting of GPR3, GPR6, and GPR12 (Uhlenbrock et al. 2002). GPR12 is known to stimulate adenylate cyclase, the enzyme responsible for cAMP production, and is expressed in human vascular endothelial cells (Uhlenbrock et al.

2002). Two endogenous ligands have been proposed for this receptor, sphingosine 1-phosphate, and tyrosol, although this was not able to be reproduced (Uhlenbrock et al. 2002; Lin et al. 2008). Sphingosine 1-phosphate is a blood-borne lipid mediator, and tyrosol is a phenethyl alcohol commonly found in olive oil (Uhlenbrock et al. 2002; Yadav et al. 2020). L-Phe bears no structural similarities to sphingosine 1-phosphate nor tyrosol; however, the latter is produced from tyrosine, a product of L-Phe oxidation, and both contain a phenol group (Chung et al. 2017; Kaufman 1999). Tyrosol has been predicted to be inactive in relation to GPCRs with a bioactive score of -0.80 determined by Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) prediction and L-Tyr did not significantly activate GPR12 in this study (Yadav et al. 2020). With this in mind, L-Phe may prove to be a more effective and potent activator of this receptor.

4.5 GPR84

GPR84 is a rather promiscuous receptor; thus, it is not surprising that it was significantly activated by the amino acid mixture. However, proposed agonists of GPR84 are typically medium-chain free fatty acids; therefore, the findings of this study are still considered novel (Suzuki et al. 2013; Wang et al. 2006). This receptor's role as a free fatty acid receptor is thought to act in conjunction with its role in immune responses as it is typically expressed in immune system-related tissues and cells (Ichimura et al. 2009). The observed amino acid activation of GPR84 by this study, emphasizes the importance of evaluating orphan receptor activity with a variety of metabolites, despite previously suggested roles due to structural characteristics of the receptor and its subfamily.

4.6 GPR88

GPR88 is primarily expressed in the CNS and is known to play a role in psychiatric disorders and the regulation of striatal functions due to its elevated levels in the striatum (Ghate et al. 2007).

The expression of this receptor is not limited to the striatum as it is also found in the cerebral cortex, amygdala, and hypothalamus (Ghate et al. 2007; Jin et al. 2014). GPR88 may also play a role in dopaminergic activity since it is highly expressed in both D1 and D2 receptor-expressing medium spiny neurons (MSNs) (Massart et al. 2009; Quintana et al. 2012). The small molecules 2-PCCA and 2-AMPP have been proposed as full agonists of GPR88, but the difficulty of identifying the natural ligand of this receptor among others found in the CNS, and targeting them pharmacologically, is in the development of brain-penetrant agonists (Jin et al. 2014, 2017). This was until the discovery of RTI-13951-33, a potent agonist of GPR88 that has no known significant off-target activity and is thought to be a possible treatment for alcoholism *via* this receptor since binding of GPR88 leads to a decrease in alcohol reinforcement and intake (Jin et al. 2018).

To our knowledge, amino acids and their derivatives have yet to be identified as agonists of GPR88, making the significant activation of this receptor by L-Phe intriguing and thus should be investigated further. L-Phe may be consumed through our diets or produced by specific bacteria in the gut microbiota (Lovenberg et al. 1962). This amino acid can be decarboxylated by the aromatic L-amino acid decarboxylase (AADC) to phenylethylamine (PEA), a ligand selective for dopamine receptors (Chen et al. 2019). PEA is found in human brain tissue and is known to act as a neuromodulator in several neuropsychiatric disorders, including depression and schizophrenia (Inwang et al. 1973; Mosnaim and Wolf 2016). This knowledge, combined with PEA's structural similarity to phenylalanine, may make it an agonist for GPR88 among other GPCRs expressed in various regions of the brain, overcoming the difficulty of crossing the blood-brain barrier.

4.7 MRGPRX2

Finally, MRGPRX2 is another promiscuous receptor, with eight proposed agonists (Alexander, et al. 2019). MRGPRX2 is present in the lumbar dorsal root ganglia with a diverse range of

proposed peptide agonists derived from endogenous proproteins, the most potent being PAMP-12 and cortistatin-14 (Kamohara et al. 2005; Robas et al. 2003). Since MRGPRX2 is a promiscuous receptor, it may prove more useful to examine which G proteins MRGPRX2 binds following activation by L-Phe. The identification of the G protein may lead to the elucidation of downstream effector proteins, and the possible effects of MRGPRX2 interaction with L-Phe. These findings can then be compared with those of other known agonists to determine if L-Phe exhibits biased signaling by selectively modulating a specific pathway.

4.8 Conclusions and future directions

This study showed that amino acids modulate the activity of orphan GPCRs, and surprisingly, L-Phe significantly activates many class A orphan GPCRs, including GPR12, GPR26, GPR84, GPR88, and MRGPRX2. Receptors were not significantly activated by the essential amino acid mixture; therefore, the activity of the orphan receptors identified in this study may also be modulated by non-essential amino acids. Future experiments should be conducted using the individual amino acids of the non-essential mixture as treatments. To characterize the activation of these receptors by L-Phe, DRCs should be conducted as well as experiments using a second assay system and a cell line that naturally expresses these receptors to determine the extent of biologically relevant activation. Receptors belonging to the same subfamilies as those significantly modulated in this study should also be treated with L-Phe to determine the specificity of this ligand.

The knowledge gained by this study helps to build a basic understanding of these orphan GPCRs and provides an avenue of therapeutic possibilities for further investigation. This study provides support for the importance of L-amino acids as extracellular signaling molecules through the activation of GPCRs. More specifically, L-Phe may prove to be more vital to metabolic processes in diseases and the overall well-being of individuals than once thought. These findings

also stress the importance of looking outside the box when attempting to identify novel agonists of these elusive orphan GPCRs as receptors of subfamilies may be differentially activated despite structural similarities.

5. References

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6. Appendix

Appendix 1. Concentration (mM) of the individual amino acids in the 50X essential amino acid mixture (Wisent, 321-010-EL), and their respective concentrations following dilution in Opti-MEM in preparation for use in treatments.

Amino acid	50X (mM)	4X (mM)	1X (mM)
<i>L</i> -Arginine HCl	36.28	2.90	0.73
<i>L</i> -Cystine 2HCl	12.91	1.03	0.26
<i>L</i> -Histidine HCl H ₂ O	13.54	1.08	0.27
<i>L</i> -Isoleucine	20.01	1.60	0.40
<i>L</i> -Leucine	19.97	1.60	0.40
<i>L</i> -Lysine HCl	24.80	1.98	0.50
<i>L</i> -Methionine	5.06	0.40	0.10
<i>L</i> -Phenylalanine	9.99	0.80	0.20
<i>L</i> -Threonine	19.98	1.60	0.40
<i>L</i> -Tryptophan	2.50	0.20	0.05
<i>L</i> -Tyrosine	9.93	0.79	0.20
<i>L</i> -Valine	19.97	1.60	0.40

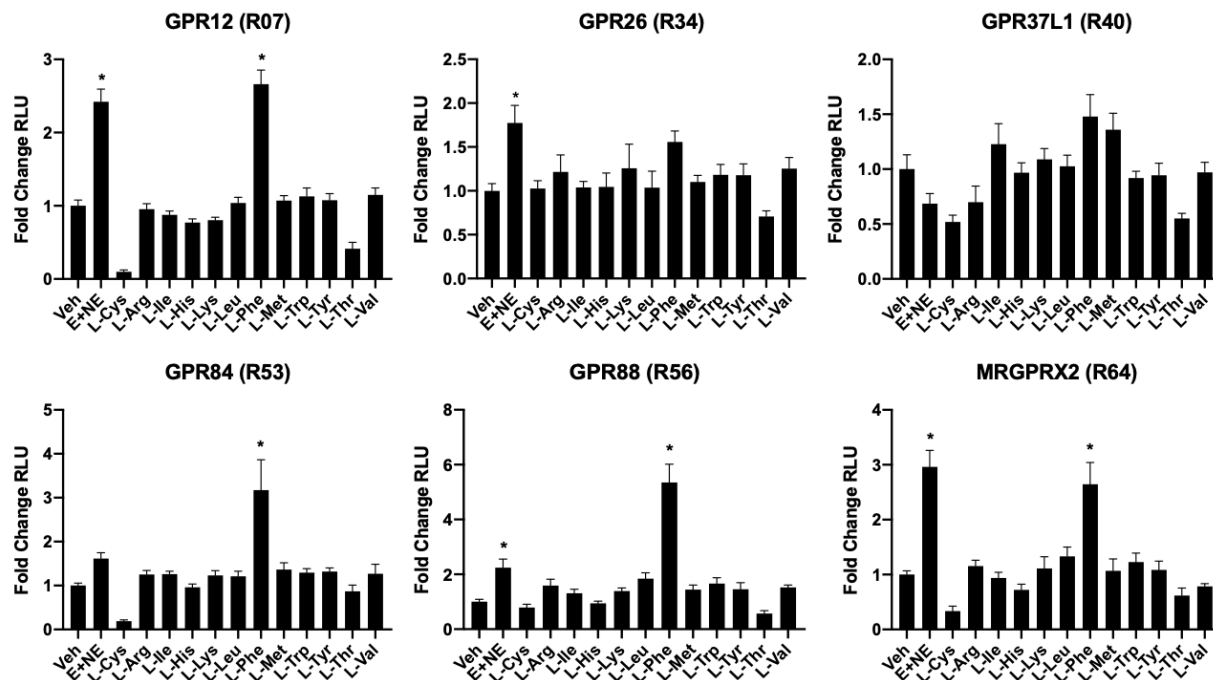
Appendix 2. Concentration (mM) of the individual amino acids in the 100X non-essential amino acid mixture (Sigma, M7145), and their respective concentrations following dilution in Opti-MEM in preparation for use in treatments.

Amino acid	100X (mM)	8X (mM)	1X (mM)
<i>L</i> -Alanine	9.99	0.80	0.10
<i>L</i> -Asparagine·H ₂ O	11.35	0.91	0.11
<i>L</i> -Aspartic acid	9.99	0.80	0.10
<i>L</i> -Glutamic acid	10.06	0.80	0.10
Glycine	9.99	0.80	0.10
<i>L</i> -Proline	9.99	0.80	0.10
<i>L</i> -Serine	9.99	0.80	0.10

Appendix 3. The relative luminescence units (RLU) and fold change relative to vehicle treatment of the activity of the 72 orphan receptors following treatment with the 4/8X essential/non-essential amino acid mixture. The significance of the fold change is determined by the p-value, with $p < 0.05$. P-values were used for the heat map in Fig. 4A.

Receptor #	Receptor name	Receptor subfamily	RLU	Fold Change	P-Value
R01	CMKOR1	Chemokine Receptors	22584.18	0.84	0.730
R02	GPER	G protein-coupled estrogen receptor	14910.84	1.08	0.863
R03	GPR101	Class A Orphans	533856.12	1.62	0.666
R04	GPR110	Adhesion Class GPCRs	6125.50	1.57	0.546
R05	GPR113	Adhesion Class GPCRs	14269.11	0.86	0.605
R06	GPR116	Adhesion Class GPCRs	13847.73	1.58	0.190
R07	GPR12	Class A Orphans	2475654.72	2.00	0.074
R08	GPR123	Adhesion Class GPCRs	8427.65	0.59	0.387
R09	GPR132	Class A Orphans	36247.32	1.16	0.279
R10	GPR133	Adhesion Class GPCRs	1654.91	0.86	0.436
R11	GPR141	Class A Orphans	116915.07	2.22	0.222
R12	GPR142	Class A Orphans	1630.97	1.05	1.000
R13	GPR143	Other 7TM proteins	10948.64	0.91	0.863
R14	GPR144	Adhesion Class GPCRs	1673.27	0.65	0.605
R15	GPR146	Class A Orphans	20702.30	0.87	0.796
R16	GPR148	Class A Orphans	4122.08	0.60	0.489
R17	GPR149	Class A Orphans	964.09	0.80	0.404
R18	GPR15	Class A Orphans	4699.20	1.13	0.948
R19	GPR150	Class A Orphans	11255.39	1.47	0.605
R20	GPR151	Class A Orphans	2123.49	1.00	0.857
R21	GPR153	Class A Orphans	8528.58	0.93	0.882
R22	GPR156	Class C Orphans	27853.14	1.29	0.480
R23	GPR160	Class A Orphans	28517.73	1.06	0.863
R24	GPR161	Class A Orphans	94094.48	1.48	0.222
R25	GPR162	Class A Orphans	5268.77	0.98	0.581
R26	GPR17	Class A Orphans	343799.07	1.19	0.796
R27	GPR171	Class A Orphans	11090.17	1.45	0.215
R28	GPR173	Class A Orphans	107043.24	1.63	0.407
R29	GPR182	Class A Orphans	1100360.78	1.50	0.283
R30	GPR19	Class A Orphans	15070.48	0.80	0.427
R31	GPR20	Class A Orphans	9322.64	1.10	0.863
R32	GPR23	Class A Orphans	255676.69	1.81	0.136

R33	GPR25	Class A Orphans	102035.78	1.73	0.546
R34	GPR26	Class A Orphans	15509.72	1.97	0.017
R35	GPR27	Class A Orphans	2981.83	1.02	0.963
R36	GPR31	Class A Orphans	12703.13	1.60	0.076
R37	GPR32	Class A Orphans	267568.25	0.65	0.297
R38	GPR35	Class A Orphans	570334.84	3.38	0.136
R39	GPR37	Class A Orphans	1202269.35	0.89	0.666
R40	GPR37L1	Class A Orphans	5784.70	0.50	0.004
R41	GPR39	Class A Orphans	16835.66	0.64	0.809
R42	GPR4	Class A Orphans	365454.51	1.11	0.489
R43	GPR45	Class A Orphans	214735.23	1.41	0.546
R44	GPR52	Class A Orphans	18334.14	1.50	0.158
R45	GPR55	Class A Orphans	27603.91	1.44	0.436
R46	GPR6	Class A Orphans	3178007.27	1.32	0.489
R47	GPR61	Class A Orphans	6965.00	0.67	0.136
R48	GPR62	Class A Orphans	7225.06	0.95	0.387
R49	GPR63	Class A Orphans	5268.77	0.96	0.606
		Adhesion Class			
R50	GPR64	GPCRs	3775.0827	1.34	0.119
R51	GPR78	Class A Orphans	3361.3805	0.89	0.743
R52	GPR83	Class A Orphans	92658.7987	1.03	0.926
R53	GPR84	Class A Orphans	4959.42	1.40	0.046
R54	GPR85	Class A Orphans	104861.46	1.26	0.114
R55	GPR87	Class A Orphans	94179.34	1.20	0.208
R56	GPR88	Class A Orphans	613744.7385	6.92	0.046
R57	KISS (GPR54)	Kissipeptin receptors	228899.6524	1.34	0.666
R58	MAS1	Class A Orphans	10674.4580	1.37	0.786
R59	MAS1L	Class A Orphans	299471.9426	0.56	0.222
R60	MRGPRD	Class A Orphans	9844.8922	0.99	1.000
R61	MRGPRF	Class A Orphans	6192.9586	1.22	0.605
R62	MRGPRG	Class A Orphans	99531.3605	0.74	0.489
R63	MRGPRX1	Class A Orphans	3322.3983	1.47	0.730
R64	MRGPRX2	Class A Orphans	4571.0741	2.85	0.040
R65	MRGPRX4	Class A Orphans	306584.3857	1.84	0.666
R66	OPN3	Opsin receptors	17328.8088	1.06	0.858
R67	OPN5	Opsin receptors	1785.5594	1.77	0.136
		Prokineticin			
R68	PK2	receptors	428470.0643	0.63	0.387
		Prolactin- Releasing			
R69	PRP	Peptide receptors	4988.2427	1.21	0.616
R70	TAAR5	Class A Orphans	20318.7023	0.80	0.511
R71	TAAR6	Class A Orphans	1112.7439	0.96	0.885
R72	TAAR9	Class A Orphans	26534.6962	0.72	0.730



Appendix 4. The fold change relative to vehicle (Veh) treatment of HTLA cells transfected with the Tango-ized orphan GPCR treated with a 4/8X essential/non-essential amino acid (E+NE) and the individual amino acids of the essential amino acid mixture. Amino acids are identified by their three-letter code. * $p < 0.05$ vs. Veh. Data are shown as mean \pm SEM.